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Comparison of *In Vitro* and *In Vivo* Screening Models for Androgenic and Estrogenic Activities

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Identification of nuclear receptor–mediated endocrine activities is important in a variety of fields, ranging from pharmacological and clinical screening, to food and feed safety, toxicological monitoring, and risk assessment. Traditionally animal studies such as the Hershberger and Allen-Doisy tests are used for the assessment of androgenic and estrogenic potencies, respectively. To allow fast analysis of the activities of new chemicals, food additives, and pharmaceutical compounds, high-throughput screening strategies have been developed. Here, a panel of mainly steroidal compounds, screened in different *in vitro* assays, was compared with two human U2-OS cell line–based CALUX® (Chemically Activated LUciferase eXpression) reporter gene assays for androgens (AR CALUX) and estrogens (ERα CALUX). Correlations found between the data of these two CALUX reporter gene assays and data obtained with other *in vitro* screening assays measuring receptor binding or reporter gene activation (CHO cell line–based) were good (correlation coefficients (r^2) between 0.54 and 0.76; $p < 0.0001$). Good correlations were also found between the *in vitro* and *in vivo* data (correlation coefficient $r^2 = 0.46$ for the AR CALUX vs. Hershberger assay and $r^2 = 0.87$ for the ERα CALUX vs. Allen-Doisy assay). The variations in the results obtained with the reporter gene assays (CALUX vs. CHO cell line based) were relatively small, showing the robustness of these types of assays. Using hierarchical clustering, bioactivity relationships between compounds but also relationships between various bioassays were determined. The *in vitro* assays were found to be good predictors of *in vivo* androgenic or estrogenic activity of a range of compounds, allowing prescreen and/or possible reduction of animal studies.

Key Words: **androgen; estrogen; CALUX; bioassay; Hershberger; Allen-Doisy.**

Steroid hormones are essential for reproduction, stress management, salt and glucose balances, as well as several other physiological processes. Due to the relatively simple chemical structure and lipophilic nature of steroids, their regulatory pathways can easily be modified by pharmacological, environmental, and/or dietary agents. Because of this, steroids or steroid-mimicking compounds are applied in many fields, making identification of the endocrine activity of these compounds important. Analytical-chemical and immunological methods are commonly used to detect steroids in food and feed, clinical practice, environmental samples, or doping control. These methods have the drawback that they only quantify the compound of interest and are not able to determine biological activity of unknown compounds or their metabolites, this in contrast to biological assays.

Bioassays in rats, mice, or rabbits were developed a long time ago to determine the endocrine activity of compounds. Important examples are the assessment of vaginal smear types to define estrogenicity (Allen and Doisy, 1923) and of the prostate, seminal vesicle, and musculus levator ani (MLA) growth to determine androgenic and anabolic activities (Hershberger *et al.*, 1953; van der Vies and de Visser, 1983). The contribution of animal studies, however, is hampered, particularly with respect to sensitivity, capacity, costs, the desire to limit animal use, and speed. To allow fast analysis of new chemicals, food additives, and pharmaceutical compounds, high throughput screening assays have been developed. These assays are based on the mechanism of action of compounds and are able to measure activation or inhibition of specific cellular pathways. These mechanism-based assays, in combination with rapid advance in automated screening technologies and bioinformatics, create new possibilities to limit animal studies. These *in vitro* detection systems are ideal for first-line screening, while positive hits can be tested more extensively using more specialized cell culture systems and animal models.

The mechanism of action of steroid hormones is well established, and opened opportunities for mechanism-based assays. Steroid hormones like estrogens and androgens are nuclear hormone receptor ligands that enter cells by diffusion where they bind to their cognate steroid receptors. Five major types of steroid receptors are known: those for estrogens, androgens, progestagens, glucocorticoids, and mineralocorticoids

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(Mangelsdorf *et al.*, 1995; McKenna and O'Malley, 2002), now classified as members of the subfamily 3 within the nuclear receptor family (Nuclear Receptors Nomenclature Committee, 1999). Upon ligand binding these receptors become activated, and they will enter the nucleus and bind to recognition sequences in promoter regions of target genes, the hormone responsive elements. Depending on the presence of receptor-interacting proteins, so-called cofactors including coactivators as well as corepressors (Chang and McDonnell, 2005; McDonnell and Norris, 2002), the DNA-bound receptor will activate transcription of the target gene, leading to new protein synthesis and an altered cellular functioning. Besides the classical genomic-based action of steroid hormones involving nuclear hormone receptors, rapid nongenomic mechanisms of steroids might also occur via putative membrane-bound receptors, at least for estrogen and progesterone signaling (Luconi *et al.*, 2004).

A whole range of so-called reporter gene assays have been developed by us and by others for compounds interacting with a range of steroid receptors, including the estrogen and androgen receptor (Balaguer *et al.*, 1999; de Gooyer *et al.*, 2003; Legler *et al.*, 1999; Schoonen *et al.*, 2000a,b; Sonneveld *et al.*, 2005; Terouanne *et al.*, 2000). In these reporter gene assays, DNA sequences containing specific hormone-responsive elements are linked to the gene of an easily measurable protein (the reporter gene; e.g., firefly luciferase). When stably introduced in a cell line expressing the cognate receptor, or by double transfection with a receptor of interest, a specific reporter cell line is generated allowing large scale screening of compounds. Similarly, simple receptor binding assays can be used to exert such screenings. However, the latter cannot distinguish between receptor interacting compounds that will lead to (partial) transcriptional activation or (partial) transcriptional inhibition of the receptor.

In this study we determined the suitability of two different reporter gene assays, either U2-OS or CHO cell line based, and two receptor binding assays using MCF-7 cells (Bergink *et al.*, 1983) as a prescreen for, or limitation of animal studies (ECVAM working group on chemicals, 2002) in determining androgenic and estrogenic activities of compounds. The *in vitro* assays (AR/ER binding and AR/ERα reporter gene assays) were found to be good predictors of AR/ER *in vivo* agonist activity of a range of mainly steroidal compounds.

MATERIALS AND METHODS

Chemicals. Androstenedione, diethylstilbestrol (DES), dexamethasone (DEX), 5α-dihydrotestosterone (DHT), 17α-estradiol, 17β-estradiol (E2), estriol, estrone, 17α-ethinyl-estradiol (EE), flutamide, genistein, levonorgestrel (LNG), 17α-methyl-testosterone (MT), mifepristone (RU486), norethynodrel (NE), progesterone, tamoxifen citrate, testosterone (T), and testosterone propionate (TP) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Methyltrienolone (R1881) was obtained from Perkin Elmer (Perkin Elmer, Groningen, The Netherlands). Cyproterone acetate (CA), medroxyprogesterone acetate (MPA), and 19-nor-testosterone (nandrolone) were obtained from Steraloids Inc. (Newport, RI). All other used compounds were supplied by the Department of Medicinal Chemistry of N.V. Organon (Oss, The Netherlands). All chemicals were diluted in either ethanol or dimethylsulphoxide (DMSO, 99.9%, Acros, Geel, Belgium) and stored at −20°C. Neomycin (G418) was purchased from Life Technologies (Breda, The Netherlands).

Animals. SPF-bred immature male and young female HSD/Cpb:ORGA rats were supplied by The Harlan Sprague-Dawley/Central Institute for the Breeding of Laboratory Animals of the Dutch Organization for Applied Scientific Research ((HSD-CPB), Zeist, The Netherlands). Rats were housed in light-, humidity- and temperature-controlled rooms (14 h light–10 h dark; 21–23°C), and given tap water and pelleted food (RMH-B, Hope farms, Linschoten, The Netherlands) *ad libitum*. Animal handling was in accordance with the Dutch law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (EU directive #86/606/CEE). The Committee for Experiments on Animals of N.V. Organon approved the experiments.

Cell culture. Human MCF-7 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supplemented with 5% fetal calf serum (FCS). U2-OS-based AR and ERα CALUX cells, stably expressing human AR and ERα and their corresponding luciferase reporter genes (e.g., multimerized responsive elements for the cognate receptor coupled to a minimal promoter element (the TATA box) and luciferase) (Legler *et al.*, 1999; Sonneveld *et al.*, 2005) were cultured in DF medium supplemented with 7.5% FCS and 200 μg/ml G418. Chinese Hamster Ovary CHO-AR and CHO-ERα cells stably expressing human AR and ERα and their corresponding luciferase reporter genes (e.g., the mouse mammary tumor virus promoter (MMTV) for AR and the rat oxytocin promoter (RO) for ERα coupled to the luciferase reporter gene), respectively, were cultured as described earlier (Schoonen *et al.*, 2000a,b).

Reporter gene assays. AR and ERα CALUX cells were plated in 96-well plates (8000 cells/well) with phenol red-free DF medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS; van der Burg *et al.*, 1988) at a volume of 200 μl per well. Two days later, the medium was refreshed, and cells were incubated with the compounds to be tested (dissolved in ethanol or DMSO) in triplicate at a 1:1000 dilution. After 24 h the medium was removed, and cells were lysed in 30 μl Triton-lysis buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min/well. For transactivation studies using CHO-derived reporter gene assays, stably transfected CHO-AR and CHO-ERα cells were used as described previously (Schoonen *et al.*, 2000a,b). Data sets for the CHO reporter gene assays were collected between 1995 and 2003 at Organon.

Receptor binding assays. For hAR and hER displacement analysis, MCF-7 cells were used. The cells were cultured, harvested, and cytosolic preparations were prepared as described previously (Schoonen *et al.*, 1995). Prior to use, cytosol equivalent to 1 g of cells was diluted with buffer at a ratio of 1:10 for hER and 1:5 for hAR (w/v). Samples were counted in a Topcount microplate scintillation counter (Perkin Elmer). The specific 50% competition level of each compound was analyzed in the range of 0.121 up to 1000 nM with a two-fold dilution range. The relative binding affinities (RBAs) of the compounds were obtained by a three-point parallel line analysis (Finney, 1978) using three subsequent concentrations in the range of 25, 50, and 75% of competition for each individual compound in relation to the reference compound. The reference compound DHT, as well as E2, was measured in the range of 0.97, 1.95, and 3.90 nM (Schoonen *et al.*, 1998, 2000a,b). Specific binding was determined by subtracting nonspecific from total binding. The mean RBA values of at least two different independent tests were calculated for each compound. The overall statistical deviation (SD) was within the 5% level. Data sets for the receptor binding assays were collected between 1983 and 2000 at Organon.

In vivo *studies.* The assay for androgenic-anabolic activity of the compounds in immature male orchidectomized rats was performed according

to the Hershberger test (Hershberger *et al.*, 1953), with minor modifications (van der Vies and de Visser, 1983). Groups of six animals per compound dose were treated subcutaneously (sc) twice a day for seven consecutive days. At the end of the treatment period autopsy was performed, and the weights of the ventral prostate, seminal vesicles, and levator ani muscle were recorded. Testosterone was used as a reference for subcutaneous administration in a dose of 160 μg/kg as the minimal active dose (MAD). The MAD was determined as the dose at which the ventral prostate weight was 1.8 times higher than the placebo value. *In vivo* activities were calculated relative to testosterone. Data sets for the Hershberger assay were collected between 1970 and 2000 at Organon. The estrogenic activity of the compounds in ovariectomized rats was determined by scoring vaginal cornification (Allen-Doisy test) as described earlier (Allen and Doisy, 1923; van der Vies and de Visser, 1983). Female adult rats were ovariectomized and primed 3 weeks later with a single dose of 1 μg estradiol (sc). One week later the reference compound estradiol and the compounds to be tested were administered (sc) with three subsequent equal doses: one dose in the afternoon of the first day, and the next two doses in the morning and afternoon of the following day. Vaginal smears were taken at the end of the third day, twice the fourth day, and again on the morning of the last day (day 5). The smears were stained with Giemsa and evaluated (de Jongh and Laqueur, 1938). For estradiol a total dosing of 0.5 μg/kg was used to obtain the minimal active dose (MAD). Each total dose is divided equally over three administrations. Test compounds are administered in total doses of 0.05 μg/rat up to 1.0 mg/rat. The usual phases observed in the morning of the 4-day estrus cycle are di-estrus (score a), pro-estrus (score e) or estrus (score g). A rat is considered to give a positive score if at least one of the smears indicates a score of e, intermediate f or g. In total six rats per compound dose were treated, and a score of 1 or 2 positive animals out of six is called weakly active, while a score of 3 up to 6 out of six animals is called active. The total dose (sc) at which 50% of the animals showed one or more positive smears is given as the minimal active dose (MAD). *In vivo* activities were calculated relative to E2. Data sets for the Allen-Doisy assay were collected between 1970 and 2000 at Organon.

Statistical analysis. Luciferase activity per well was measured as relative light units (RLUs). Fold induction was calculated by dividing the mean value of light units from exposed and nonexposed (solvent control) wells. For CALUX cells, luciferase induction as a percentage of maximal DHT (AR CALUX) or E2 (ERα CALUX) activity was calculated by setting the highest fold induction of DHT or E2 at 100%. Data are represented as mean values ± SEM from at least three independent experiments with each experimental point performed in triplicate. Dose-response curves were fitted using the sigmoidal fit $y = (a_0 + a_1)/(1 + \exp[-(x - a_2)/a_3])$ in GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA), which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). The EC50 values were calculated by determining the concentration by which 50% of maximum activity was reached using the sigmoidal fit equation. At least eight different concentrations covering the total S-curve were included for each compound. The relative transactivation activity (RTA) of each compound tested was calculated as the ratio of maximal luciferase reporter gene induction values of each compound and the maximal luciferase reporter gene induction value of reference compound of the specific assay. The transactivation activity of the reference compounds DHT or E2 was arbitrarily set at 100. The relative agonistic activities (RAA) for the CALUX reporter gene assays were calculated by dividing the EC50 concentration of the reference compound with the EC50 concentration of the compound of interest. Relative agonistic activity studies with CHO reporter gene assays were carried out with five concentrations of the standards DHT and E2 at 1.50×10^{-11}, 3.00×10^{-11}, 6.00×10^{-11}, 1.21×10^{10}, and 2.42×10^{-10} M and three subsequent concentrations of the compound of interest in the range of 1 pM up to 100 nM. The relative agonistic activities of the compounds were obtained by a 3-point parallel line analysis (Finney, 1978) using three up-following concentrations in the range of 25, 50, and 75% activation for each individual compound in relation to the reference compound (Schoonen *et al.*, 1998, 2000a,b). The mean RAA values were calculated from at least two different independent tests. The overall SD was within the 5% level. In the *in vivo* Allen-Doisy and Hershberger tests, the mean scores per dose were calculated. The RAA values for *in vivo* compound testing were calculated by dividing the MAD of the standards testosterone (Hershberger assay) or E2 (Allen-Doisy assay) by the MAD of the compound of interest. Correlation coefficients (r^2) and their correspondent *p*-values were calculated with GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA). Cutoff values were 0.0001 for Allen-Doisy comparison, and 0.001 for reporter gene, receptor binding, and Hershberger comparisons. Two-dimensional hierarchical clustering on the base 10 logarithm of the RAA data was performed using the correlation option within the clustergram function from the bioinformatics toolbox in Matlab (The Mathworks, the Netherlands).

RESULTS

Comparison of Different In Vitro *Reporter Gene Assays for Determination of Androgenic and Estrogenic Activities*

The results obtained in two different laboratories were compared by using a panel of mainly steroidal chemicals with *in vitro* reporter gene assays for androgen and estrogen receptors. The AR and ERα CALUX cell lines as well as the CHO cell lines are efficient tools to screen for agonistic and antagonistic effects of compounds toward the androgen receptor and estrogen receptor alpha, respectively (Schoonen *et al.*, 2000a,b; Sonneveld *et al.*, 2005). AR and ERα CALUX cells are human U2-OS cell line based with the same basal characteristics as other CALUX reporter gene assays (ERβ, PR, and GR CALUX), being robust, easy maintainable, stable and strongly responsive, and selective. The range in EC50 values measured with different ligands over time, including the positive controls DHT (AR CALUX) and E2 (ERα CALUX), is small, reflected by an inter-assay CV of 22% for the AR CALUX and 25% for the ERα CALUX reporter gene assay (Sonneveld *et al.*, 2005).

Typical dose-response curves for several natural as well as synthetic androgens and estrogens using AR and ERα CALUX reporter gene assays, respectively, are shown in Figure 1. The AR CALUX cell line showed high sensitivity toward all androgens tested (Fig. 1A and Table 1), with the following range of potencies (EC50 values): dihydrotestosterone (DHT; 110 pM), testosterone (T; 657 pM), and its 19-nor derivatives nandrolone (19-nor-T; 301 pM), 19-nor-11-keto-T (2845 pM), and 11-methylene-19-nor-T (98 pM). The selectivity of the AR CALUX cells is high, since representative steroids for other hormone receptors (E2 and EE for ER, progesterone for PR, and dexamethasone for GR) showed no substantial agonistic response, with relative agonistic activities below 0.001, except for dexamethasone (0.003), which additionally has a relative transcriptional activity (RTA) of 8% compared to DHT (see Table 1; Sonneveld *et al.*, 2005).

In line with the AR CALUX cell line, the ERα CALUX cell line showed high sensitivity toward all estrogens tested, with the following range of potencies (EC50 values): 11β-ethenyl-E2 (4 pM), E2 (16 pM), 3β-OH-5α-hydrogen-11β-ethenyl-NET (86 pM), 19-nor-5α-NET (18000 pM), and norethisterone

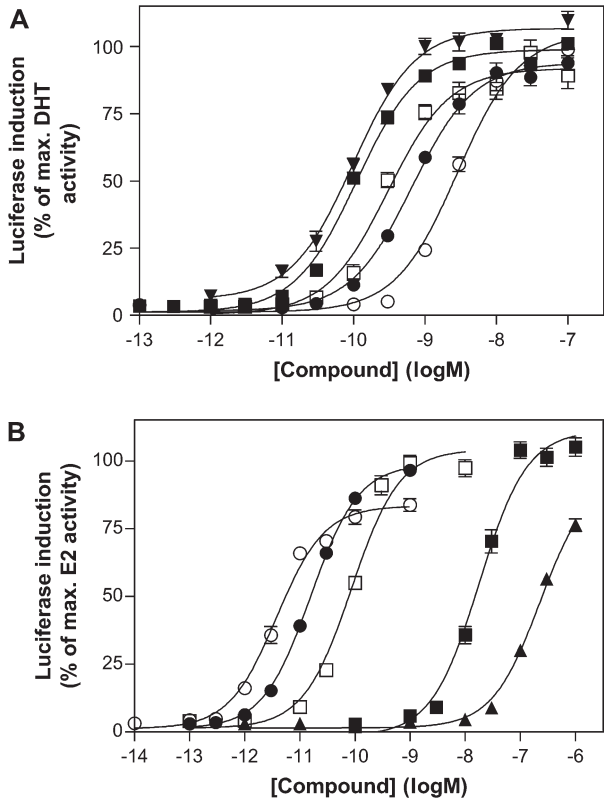

FIG. 1. Dose response curves for different receptor activating compounds in the AR and ERα CALUX reporter gene assays. AR and ERα CALUX cells were plated in 96-well plates. AR CALUX cells (A) were treated with the androgenic compounds DHT (■), testosterone (●), and its 19-nor derivatives 19-nor-11-keto-T (○), 11-methylene-19-nor-T (▼), and 19-nor-T (□), and ERα CALUX cells (B) were treated with the estrogenic compounds E2 (●), 11β-ethenyl-E2 (○), NET (▲), 19-nor-5α-NET (■), and 3β-OH-5α-hydrogen-11β-ethenyl-NET (□) for 24 h using DF medium containing 5% DCC-FCS. Each point represents the mean of at least three independent experiments ± SEM.

(NET; 189234 pM) (Fig. 1B and Table 2). As shown again, minor steroid modifications greatly influence activity on the ERα. Furthermore, the ERα CALUX cells showed high selectivity toward estrogens, since representative steroids for other hormone receptors (testosterone and DHT for AR, progesterone for PR and dexamethasone for GR) showed no substantial agonistic response (relative agonistic activities below 0.0001) (Table 2).

The CHO-hAR and CHO-hERα cell lines used in this study are members of a panel of CHO-derived steroid reporter cell lines containing hERβ, hPR, and hGR as well (Schoonen *et al.*, 2000a,b). The data generated at Organon originate from a historical database, aimed at pharmaceutical applications and dosages. For this reason, these data focused on relatively high-potency compounds, allowing identification of compounds that have EC50 values not lower than 0.001 times of the reference compounds. Therefore, the relative potency cutoff value for the correlation determination between AR or ERα CALUX reporter gene assays and their corresponding CHO reporter gene counterparts was set at 0.001, and relative agonistic activities below this value were not included in the correlation analysis. Furthermore, known AR antagonists (flutamide, cyproterone acetate, RU486) and ERα antagonists (tamoxifen, raloxifen, ICI 164.384) also were excluded in the correlation since, although these compounds can bind to their cognate receptor, they generally fail in transactivating, but can be identified in an antagonistic setting (data not shown). Also compounds for which no exact RAA was determined (indicated as < or > in Tables 1 and 2) were excluded from the correlation.

Despite the different setups at the two laboratories, surprisingly good correlations were found between data obtained with the same compounds in the different reporter gene assays. Figure 2 shows the correlation between data obtained between the AR CALUX cells and CHO-AR cells (Fig, 2A: 47 compounds tested; $r^2 = 0.75$; $p < 0.0001$), and both ERα-expressing cell lines (Fig. 2B: 36 compounds tested; $r^2 = 0.74$; $p < 0.0001$). These correlation coefficients were very similar to the correlation coefficients found in an unbiased approach where no correlation exclusions were performed (comparison between both AR reporter gene assays: $n = 60$, $r^2 = 0.76$; ERα reporter gene assays: $n = 61$, $r^2 = 0.78$; data not shown). This clearly shows that the data restrictions for correlation have no influence on the correlation coefficients. Notable differences between the AR CALUX and CHO-AR reporter gene assays (more than 10 times difference between RAAs; see Table 1) were: 17α-(2-propenyl)-19-nor-T, 11β-ethyl-NET, Δ15-NET and T-17, 17′-(2, 2′-oxybisacetate) (all AR CALUX > CHO-AR). Notable differences between the ERα CALUX and CHO-ERα reporter gene assays (more than 10 times difference between RAAs; see Table 2) were the natural estrogens estriol and estrone (both ERα CALUX < CHO-ERα) and 17α-iodovinyl-E2 (*E*) (ERα CALUX > CHO-ERα).

Because the CALUX datasets for both AR and ERα were the most extensive, we focused on these datasets only and compared these with the receptor binding assays and *in vivo* assays.

Comparison of Reporter Gene Assays with Receptor Binding Assays

The results of reporter gene assays were compared with those of receptor binding assays. As for the comparison between the different reporter gene assays, the relative potency cutoff value for the correlation determination between RAAs from AR or ERα CALUX reporter gene assays and their corresponding relative binding activities (RBAs) derived from AR and ER binding assays was set at 0.001. Figure 3 shows that correlations found between the receptor binding and CALUX reporter gene assays were excellent. When comparing AR binding capacities of various androgens (43 compounds, see Table 1) with transactivation potential of these compounds in the AR CALUX reporter gene assay, a correlation coefficient of

TABLE 1
AR CALUX $LogEC_{50}$ Values, Relative Transcriptional Activity (RTA), and Relative Agonistic Activity (RAA)

	Modification						AR CALUX					
Compound	R3	R4–5	R6	R7	R11	R17	$LogEC_{50}$(M)	RTA (%)	RAA 1 = DHT	CHO-AR RAA 1 = DHT	AR binding RBA 1 = DHT	Hershberger RAA 1 = T
Nandrolone derivatives												
19-nor-T (Nandrolone)							−9.5	92	0.486	0.530	0.474	0.080
6α-methyl-NET			e			i	−8.9	50	0.139	0.021	0.083	nd
6α-methyl-19-nor-T			e				−9.6	127	0.464	1.256	0.895	>0.064
MENT = 7α-methyl-19-nor-T				e			−10.1	121	1.983	2.630	1.405	1.280
7α-methyl-NET				e		i	−9.7	78	0.912	0.206	0.291	0.500
7α-methyl-11β-methyl-NET				e	e	i	−9.4	69	0.397	0.166	0.253	0.008
7α-methyl-11-methylene-NET				e	f	i	−9.1	74	0.218	0.064	0.133	0.008
7α-methyl-11-ethylene-NET				e	g	i	−8.5	69	0.054	0.016	0.097	0.250
7α-methyl-17α-(2-propenyl)-19-nor-T				e		o	−9.7	79	0.956	0.149	0.381	0.016
11α-OH-19-nor-T					l		−7.7	100	0.008	0.012	0.008	0.002
11β-OH-19-nor-T					m		−7.3	97	0.003	0.003	0.003	0.006
11β-methyl-19-nor-T					e		−9.6	120	0.734	1.200	0.620	<0.123
11-methylene-19-nor-T					f		−10.0	107	1.669	2.003	0.930	0.055
11β-ethyl-NET					h	i	−8.0	48	0.017	0.003	0.020	0.032
11β-ethinyl-NET					i	i	−8.5	64	0.051	0.020	0.046	0.051
11β-ethenyl-NET					k	i	−7.1	36	0.002	0.004	0.021	0.005
11-keto-19-nor-T					n		−8.6	102	0.059	0.065	0.109	0.007
17α-methyl-19-nor-T						e	−9.9	87	1.301	0.815	0.380	>0.032
Norethisterone (NET)						i	−8.2	69	0.026	0.011	0.034	0.064
17α-(2-propenyl)-19-nor-T						o	−8.4	51	0.047	0.003	0.111	0.008
3-deoxy-11β-OH-19-nor-T	a				m		−8.4	88	0.042	0.000	nd	0.032
3-deoxy-11-keto-17α-ethyl-19-nor-T	a				n	h	−8.0	44	0.017	0.000	nd	0.032
5α-hydrogen-7α-methyl-NET		b		e		i	−9.3	64	0.313	0.038	0.307	0.127
5α-hydrogen-11β-methyl-NET		b			e	i	−8.7	52	0.092	0.027	0.128	nd
5α-hydrogen-11β-ethyl-NET		b			h	i	−8.0	45	0.018	0.002	0.032	0.008
5α-hydrogen-11β-ethinyl-17α-ethenyl-19-nor-T		b			i	k	−8.7	53	0.092	0.040	0.025	nd
5α-hydrogen-11β-ethinyl-NET		b			i	i	−8.5	55	0.051	nd	0.070	0.016
5α-hydrogen-19-nor-T		b					−9.4	110	0.405	0.259	0.588	<0.008
5α-hydrogen-NET		b				i	−8.5	149	0.056	0.008	0.051	0.032
5α-hydrogen-17α-(2-propenyl)-19-nor-T		b				o	>−6.0	23	0.007	0.001	0.025	0.008
Δ15-NET		c				i	−8.3	57	0.036	0.001	0.025	0.043
7α-methyl-androst-5(10)-ene-19-nor-T		d		e			−9.4	102	0.466	0.809	nd	0.160
Norethynodrel (NE)		d				i	−8.2	48	0.027	0.006	0.007	0.008
Testosterone derivatives												
T							−9.2	94	0.146	0.168	0.171	1.000
T propionate						p	−9.1	81	0.200	nd	nd	>1.000
Testosterone derivatives (continued)												
11-methylene-17β-propionate-T					f	p	−8.7	86	0.080	0.017	nd	1.000
17α-methyl-T						e	−9.1	108	0.197	0.195	0.206	>1.000
T-17,17′-(2,2′-oxybisacetate)						p	−9.3	81	0.319	0.014	nd	0.032
7α-methyl-T				e			−8.5	87	0.051	0.455	0.250	nd
5α-hydrogen-T(DHT)		b					−9.9	100	1.000	1.000	1.000	>0.250
(14β,17α,20S)-20-OH-19-norpregna-4,9-diene-3-one							−9.7	90	0.975	0.530	0.400	0.127
(11β,14β,17α,20S)-11-ethenyl-20-OH-19-norpregna-4,9-diene-3-one							−9.4	82	0.453	0.345	0.405	0.127
Androst-4-ene-3,17-dione							−8.4	82	0.057	nd	0.001	nd
R1881							−9.9	69	1.063	0.976	0.595	nd
Flutamide							>−5.0	<5	0.000	<0.001	0.015	0.002
17β-mercapto-androst-4-en-3-one							>−6.0	41	0.000	<0.001	0.028	0.032
11β-ethenyl-19-nor-androstenedione					k		−8.3	71	0.036	0.026	nd	0.064
5α-Androstane-3α,17β-diol							−7.7	66	0.009	0.003	nd	0.500

TABLE 1—*Continued*

Compound	Modification					AR CALUX			CHO-AR	AR binding	Hershberger
	R3	R4–5	R6	R7	R11	R17	$LogEC_{50}$(M)	RTA (%)	RAA 1 = DHT	RAA 1 = DHT	RBA 1 = DHT	RAA 1 = T
Progestagens												
Progesterone							>−5.0	36	0.000	<0.001	0.019	0.064
Norgestimate							−5.9	25	0.000	<0.002	0.027	nd
MPA							−8.2	75	0.014	0.042	0.197	0.032
LNG							−8.6	79	0.074	0.051	0.105	0.250
LNG acetate							−9.1	59	0.222	0.061	nd	0.250
Cronolon							>−6.0	12	0.000	<0.002	0.010	nd
CA							−5.4	50	0.001	<0.001	0.067	0.002
11-methylene-17α-azidomethyl-18-methyl-19-nor-T	f						−9.3	73	0.333	0.210	nd	0.500
11-methylene-17β-spiro-(Estr-4-ene-17,2′(3′H)furan)-3-one	f						−8.6	59	0.076	nd	0.053	0.032
RU 486 = Mifepristone							>−5.0	<5	0.000	0.000	0.028	nd
Estrogens												
EE						i	>−5.0	<5	0.000	<0.001	0.003	nd
E2							−5.5	93	0.000	<0.001	0.038	nd
17α-estradiol							>−5.0	<5	0.000	<0.001	0.034	>0.008
Allylestrenol							>−5.0	<5	0.000	<0.001	0.019	0.002
7α-methyl-17α-(2-propenyl)-estr-5(10)-en-17β-ol							−9.2	71	0.250	0.064	nd	0.032
Glucocorticoid												
Dexamethasone							−7.3	8	0.003	<0.001	0.001	<0.032

Note. $LogEC_{50}$ values, Relative Transcriptional Activity (RTA), and Relative Agonistic Activity (RAA) of various compounds in the AR CALUX reporter gene assay, and their corresponding RAA and Relative Binding Activity (RBA) in the CHO-AR reporter gene assay, AR binding assay, and Hershberger assay. nd = not determined. Abbreviations: a = 3-deoxy; b = 5α-hydrogen; c = Δ15; d = Δ5–10; e = methyl; f = methylene; g = ethylene; h = ethyl; i = ethinyl; k = ethenyl; l = α-hydroxy; m = β-hydroxy; n = keto; o = 2-propenyl; p = propionate; R3 = 3-position; R4–5 = 4–5-position; R6 = 6α-position; R7 = 7α-position; R11 = 11-position; R17 = 17-position.

$r^2 = 0.74$ ($p < 0.0001$) was found (Fig. 3A). Some compounds were found that showed relatively high binding activity coupled to absent or low transactivation potential (Table 1). These compounds were known full (flutamide) or partial (cyproterone acetate; CA) AR antagonists and were therefore not included in the correlation calculation. Also other compounds showing absent or low AR transactivation activity (relative potencies below 0.001 and therefore not included in correlation) were found to bind the receptor with moderate affinity: E2, 17α-estradiol, progesterone, norgestimate, allylestrenol, and RU 486 (Table 1). On the other hand, the relatively high potency of the testosterone precursor androstenedione in the AR CALUX cell line was obvious, while binding to the androgen receptor was low, indicating the metabolic capacity generating more active compounds in U2-OS (17β-HSD activity).

In the comparison of ERα binding data with ERα transactivation data obtained for various estrogens (34 compounds, see Table 2), a correlation coefficient of $r^2 = 0.54$ ($p < 0.0001$) was found (Fig. 3B). As for the AR, ERα antagonists (tamoxifen, raloxifen and ICI 164.384) also were relatively strong receptor binders while showing no transactivation potential (Table 2) and were not included in the calculation. Of the 34 included compounds, three compounds showed over 10-fold differences in favor of ER binding: genistein (15×), 3β-OH-11β-ethyl-NET (16×), 3α-OH-5α-hydrogen-11β-ethyl-NET (12×), and 3β-OH-5α-hydrogen-11β-ethyl-NET (41×), suggesting these may be weak transactivators for ERα, as at higher dosages they still give full transactivation.

In addition, comparably good correlations were found using CHO-derived reporter gene data and receptor binding data for both AR ($r^2 = 0.86$; $n = 38$; $p < 0.0001$) and ERα ($r^2 = 0.69$; $n = 43$; $p < 0.0001$) (data not shown).

Comparison of Reporter Gene Assay Data Sets with in Vivo *Assay Data Sets*

To test whether reporter gene assays can be used as prescreens or reduction of *in vivo* assays for hormonal activity, we compared the AR and ERα CALUX data sets with *in vivo* Hershberger and Allen-Doisy assays, respectively (Fig. 4; Tables 1 and 2). The relative potency cutoff value for the RAAs used in the correlation determination between the AR CALUX reporter gene assay and the corresponding RAAs from the Hershberger assay was set at 0.001, while the cutoff value for the RAAs used in the comparison between the ERα CALUX reporter gene assay and the Allen-Doisy assay was set at 0.0001 due to a better dynamic determination range for

TABLE 2
ERα CALUX $LogEC_{50}$ Values, Relative Transcriptional Activity (RTA), and Relative Agonistic Activity (RAA)

	Modification						ERα CALUX					
Compound	R3	R4–5	R6	R7	R11	R17	$LogEC_{50}$ (M)	RTA (%)	RAA 1 = E2	CHO-ERα RAA 1 = E2	ER binding RBA 1 = E2	Allen-Doisy RAA 1 = E2
Estradiol derivatives												
E2 (17β-Estradiol)							−10.8	100	1.0000	1.0000	1.0000	1.0000
Estriol							−9.9	100	0.0355	0.3020	0.0650	0.0156
Estrone							−9.0	119	0.0159	0.2210	0.0670	0.1250
11-methylene-E2					f		−10.9	91	0.9947	0.9150	1.6000	1.3300
11β-methyl-E2					e		−11.2	109	2.7473	0.7050	0.9020	nd
11β-ethenyl-E2					k		−11.4	84	3.1584	1.0450	1.3200	2.0000
11β-ethinyl-E2					i		−11.1	85	1.6830	0.6950	0.4500	1.5000
17α-estradiol							−8.8	104	0.0120	0.0610	0.0740	0.0062
EE derivatives												
EE (17α-ethinyl-E2)						i	−11.1	92	1.8600	0.7780	1.2090	1.3333
11-methylene-EE					f	i	−11.2	83	1.7469	0.6490	1.8400	1.0000
11β-methyl-EE					e	i	−11.2	85	2.0771	0.7100	1.7860	1.3300
11β-ethyl-EE					h	i	−11.2	87	1.7783	0.5470	1.8010	2.0000
11β-ethinyl-EE					i	i	−11.0	90	1.4567	0.4980	0.9080	nd
11β-chloromethyl-EE					u	i	−11.0	75	1.2584	0.3950	0.9450	1.3300
Mestranol (3-methoxy-EE)	r					i	−8.5	76	0.0045	nd	nd	0.2000
17α-iodovinyl-E2 (*E*)						v	−9.9	126	0.1202	0.0010	0.4700	nd
Non-aromatic estradiol												
3β-OH-11-methylene-NET					f	i	−9.5	111	0.0449	0.0230	0.1660	nd
3β-OH-11β-ethenyl-NET					k	i	−10.3	93	0.1820	0.0880	0.3450	0.1250
3β-OH-11β-ethyl-NET					h	i	−9.5	79	0.0399	0.1130	0.6200	0.5000
3β-OH-7α-methyl-NET				e		i	−10.3	85	0.2532	0.1920	0.3080	0.0080
3β-OH-5α-hydrogen-NET		b				i	−9.2	119	0.0155	0.0250	0.0780	0.0003
3β-OH-5α-hydrogen-11-methylene-NET		b			f	i	−9.2	129	0.0240	0.0620	0.1550	nd
3β-OH-5α-hydrogen-11β-methyl-NET		b			e	i	−7.7	101	0.0626	0.0500	0.2330	0.0330
3β-OH-5α-hydrogen-11β-ethenyl-NET		b			k	i	−10.1	104	0.1175	0.1650	0.4900	0.0625
3β-OH-5α-hydrogen-11β-ethyl-NET		b			h	i	−9.5	91	0.0350	0.1130	1.4200	0.2500
3β-OH-5α-hydrogen-7α-methyl-NET		b		e		i	−9.7	101	0.0598	0.0690	0.2070	0.0023
3α-OH-11-methylene-NET	s				f	i	−8.2	110	0.0024	0.0050	0.0070	nd
3α-OH-11β-ethyl-NET	s				h	i	−9.0	79	0.0133	0.0370	0.0395	0.0210
3α-OH-11β-ethinyl-NET	s	b			i	i	−9.3	95	0.0284	0.0750	0.0480	nd
3α-OH-7α-methyl-NET	s			e		i	−8.8	83	0.0076	0.0310	0.0530	0.0010
3α-OH-5α-hydrogen-11β-ethyl-NET	s	b			h	i	−8.9	97	0.0103	0.0310	0.1190	0.0830
3α-OH-5β-hydrogen-7α-methyl-NET	s	b"		e		i	−7.8	105	0.0010	0.0010	0.0030	nd
Estrogenic compounds												
DES							−10.4	98	0.2138	0.4670	1.7400	0.5000
Genistein							−7.3	135	0.0002	0.0010	0.0030	0.0005
Antiestrogens												
Tamoxifen (citrate)							>−5.0	<5	0.0000	0.0033	0.0010	0.0013
Clomiphene citrate							−8.5	31	0.0050	0.0010	0.1920	0.0010
Nafoxidine							>−5.0	5	0.0000	nd	nd	>0.0005
ICI 164.384							>−5.0	<5	0.0000	0.0010	0.0370	0.0001
Raloxifen							>−6.0	5	0.0000	0.0010	0.2500	0.0001
3-keto-steroids												
NE	t	d				i	−9.1	99	0.0179	0.0030	0.0060	0.0020
Testosterone	t						>−5.0	12	0.0000	0.0010	nd	0.0001
5α-hydrogen-T (DHT)	t	b					>−6.0	<5	0.0000	0.0010	0.0010	>0.0005
19-nor-T (Nandrolone)	t						−6.7	94	0.0001	0.0010	nd	>0.0005

TABLE 2—*Continued*

Compound	Modification						ERα CALUX			CHO-ERα	ER binding	Allen-Doisy
	R3	R4–5	R6	R7	R11	R17	$LogEC_{50}$ (M)	RTA (%)	RAA 1 = E2	RAA 1 = E2	RBA 1 = E2	RAA 1 = E2
5α-hydrogen-19-nor-T	t	b					−6.5	125	0.0001	0.0010	nd	nd
11β-methyl-19-nor-T	t				e		−6.5	98	0.0001	0.0010	nd	nd
11-keto-19-nor-T	t				n		>−5.0	<5	0.0000	0.0010	nd	nd
R1881	t						−6.2	61	0.0000	0.0010	nd	nd
Progestagens												
Progesterone	t						>−6.0	<5	0.0000	<0.0002	0.0060	nd
17α-Progesterone	t					w	>−5.0	<5	0.0000	0.0010	0.0010	nd
NET	t					i	−6.7	88	0.0001	0.0010	0.0030	0.0001
MPA	t		e				>−6.0	<5	0.0000	0.0010	nd	nd
CA	t						>−6.0	<5	0.0000	0.0010	nd	nd
LNG	t					i	−5.8	92	0.0000	0.0010	0.0020	0.0001
Norgestimate	t						>−5.0	<5	0.0000	0.0010	nd	nd
Cronolon	t						−6.2	54	0.0000	0.0010	0.0010	nd
6α-methyl-19-nor-T	t		e				−5.3	94	0.0000	0.0010	nd	nd
7α-methyl-19-nor-T (MENT)	t			e			−7.6	96	0.0007	0.0014	nd	0.0004
6α-methyl-NET	t		e			i	>−5.0	16	0.0000	0.0010	nd	nd
7α-methyl-NET	t			e		i	−7.4	107	0.0004	0.0010	0.0010	0.0020
5α-hydrogen-11β-methyl-NET	t	b			e	i	−7.9	117	0.0013	0.0067	0.0048	nd
5α-hydrogen-NET	t	b				i	−7.9	101	0.0012	0.0043	0.0020	0.0003
Antiprogestagen												
RU 486 (Mifepristone)	t						−5.9	31	0.0000	0.0010	0.0010	nd
Glucocorticoid												
Dexamethasone	t						>−5.0	<5	0.0000	0.0010	0.0010	>0.0005

Note. $LogEC_{50}$ values, Relative Transcriptional Activity (RTA), and Relative Agonistic Activity (RAA) of various compounds in the ERα CALUX reporter gene assay, and their corresponding RAA and Relative Binding Activity (RBA) in the CHO-ERα reporter gene assay, ER binding assay, and Allen-Doisy assay. nd = not determined. Abbreviations: b = 5α-hydrogen; b″ = 5β-hydrogen; d = Δ5–10; e = methyl; f = methylene; h = ethyl; i = ethinyl; k = ethenyl; n = keto; r = 3-methoxy; s = 3α-hydroxy; t = 3-keto; u = chloromethyl; v = 17α-iodovinyl; w = 17α-hydroxy; R3 = 3-position; R4–5 = 4–5-position; R6 = 6α-position; R7 = 7α-position; R11 = 11-position; R17 = 17-position.

the latter *in vivo* assay. Besides the exclusion of compounds with RAAs below the cutoff values, compounds for which no exact RAA was determined (indicated as < or > in Tables 1 and 2) were also excluded from the correlation.

The correlation of AR CALUX data with those of the Hershberger assay was lower than found with the other AR *in vitro* assays (Fig. 4A); comparison of 34 compounds resulted in a correlation coefficient of $r^2 = 0.46$ ($p < 0.0001$). Some notable exceptions were found that showed relatively high *in vitro* reporter gene activity coupled to low *in vivo* potential: nandrolone (6×), 11-keto-19-nor-T (7×), (14β,17α,20S)-20-OH-19-norpregna-4,9-diene-3-one (8×); and 7α-methyl-17α-(2-propenyl)-estr-5(10)-en-17β-ol (8×). Also, exceptions were found that showed relatively high *in vivo* activity coupled to low *in vitro* reporter gene potential, for example, testosterone (seven times difference).

The correlation of ERα CALUX data with those of Allen-Doisy assay testing 31 compounds was excellent ($r^2 = 0.87$; $p < 0.0001$) (Fig. 4B). In this *in vitro*/*in vivo* comparison, exceptions were found that showed relatively high *in vitro* reporter gene activity and low *in vivo* potential: 3β-OH-7α-methyl-NET (32×), 3β-OH-5α-hydrogen-7α-methyl-NET (26×), and 3β-OH-5α-hydrogen-NET (52×). Again, exceptions were found that showed relatively high *in vivo* activity coupled to low *in vitro* reporter gene activity: 3β-OH-11β-ethyl-NET (12 times difference) and mestranol (44×). In addition, comparable correlations were found using CHO-derived reporter gene data and Hershberger and Allen-Doisy data for both AR ($r^2 = 0.47$; $n = 31$; $p < 0.0001$) and ERα ($r^2 = 0.78$; $n = 32$; $p < 0.0001$) (data not shown).

Compound Activity Profiling Using Multiple Assays

The large steroid compound data set obtained with various *in vitro* and *in vivo* assays (see Tables 1 and 2) allows the cross-assay bioactivity profiling of a given compound. By means of hierarchical clustering, bioactivity relationships between both compounds and between various bioassays can be determined. Furthermore, these relationships can be visualized easily by coloring the activity of a compound; the higher the activity of the compound in a certain assay, the more intense the red color (see Fig. 5). Since our interest was in the evaluation of *in vitro* assays as prescreen for *in vivo* screens, we concentrated on the trends rather than on the absolute numbers. For this reason, the hierarchical clustering was performed on the base 10 logarithm of the RAA data.

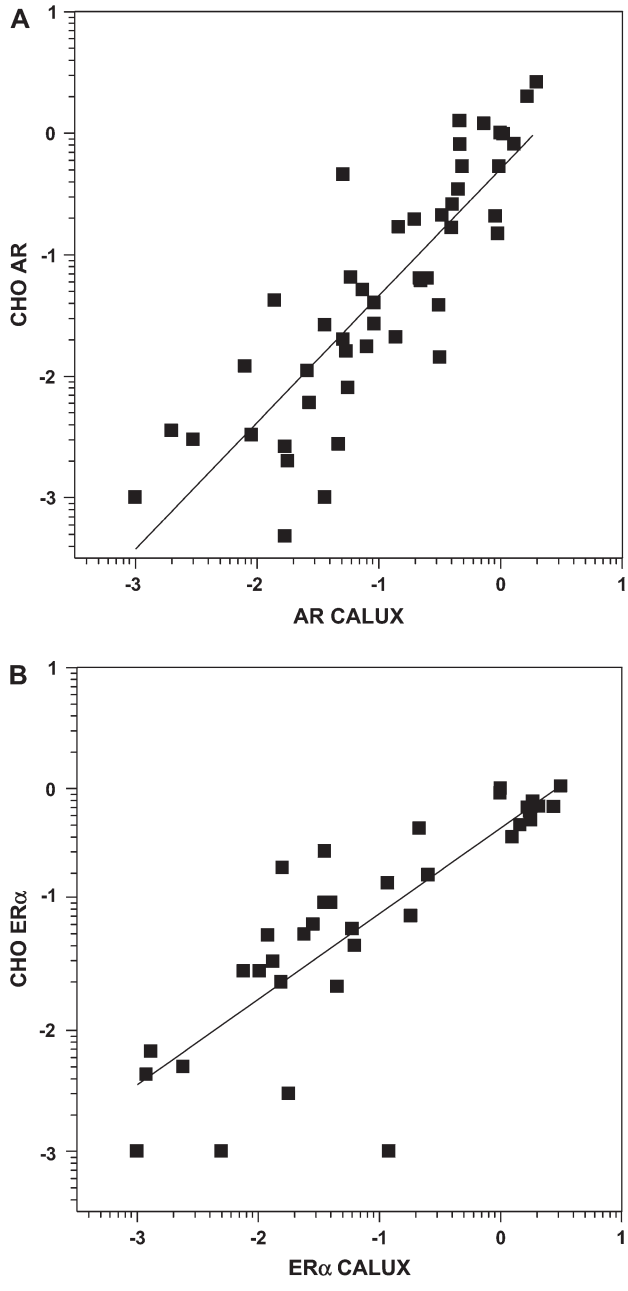

FIG. 2. Comparison of different *in vitro* reporter gene assays for determination of androgenic and estrogenic activity of various compounds. Relative Agonistic Activities (RAAs) of various androgenic or estrogenic compounds (see Tables 1 and 2) were determined by AR and ERα CALUX reporter gene assays and compared with their corresponding RAAs obtained with CHO-AR (A) and CHO-ERα (B) reporter gene assays, respectively.

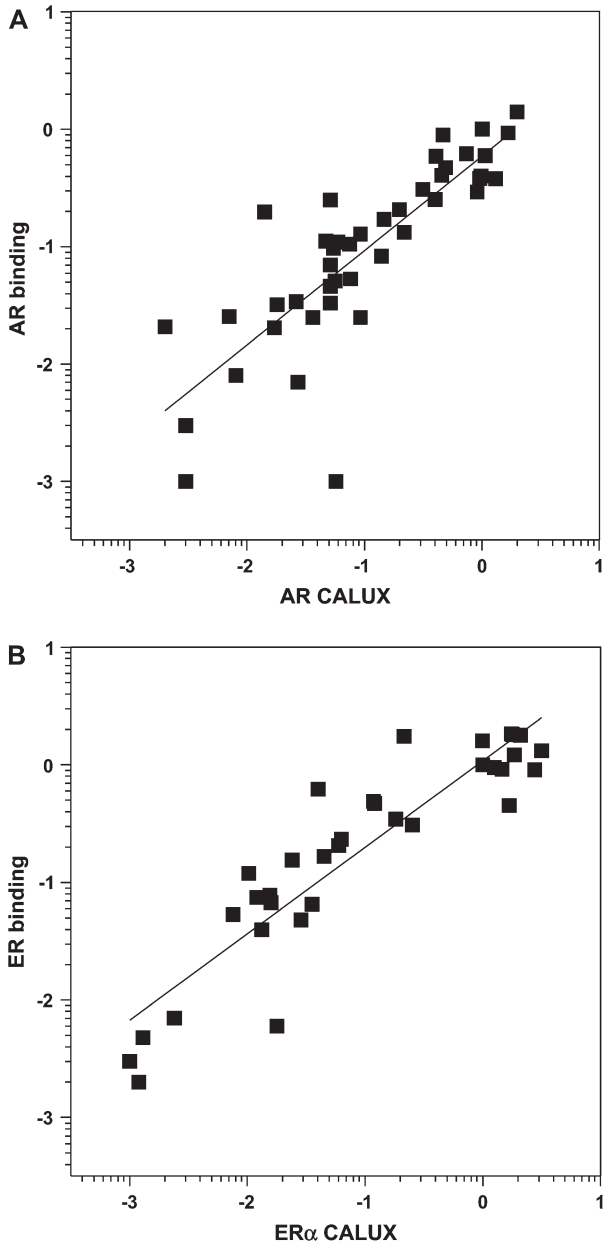

FIG. 3. Comparison of *in vitro* CALUX reporter gene assays with *in vitro* receptor binding assays for determination of androgenic and estrogenic activity of various compounds. Relative Agonistic Activities (RAAs) of various androgenic or estrogenic compounds (see Tables 1 and 2) were determined by AR and ERα CALUX reporter gene assays and compared with the relative binding activities (RBAs) obtained with AR (A) and ER (B) receptor binding assays, respectively.

Although the dataset of androgenic or estrogenic compounds tested in all four assays is rather limited (only those compounds included that in all correlations were used; $n = 26$ for the androgens and $n = 30$ for the estrogens), already interesting relationships in both directions (compounds and assays) could be observed (Fig. 5). For the androgenic compounds, the compounds with 11β-substitutions, 5α-hydrogen, or 7α-methyl substitutions tend to cluster separately (Fig. 5A). For the estrogenic compounds, the structure relationships are more evident; the groups of E2-derivatives, EE-derivatives, and NET-derivatives with 3β-OH/5α-hydrogen substitutions clearly cluster separately (Fig. 5B). Besides these compound relationships, relationships between the different assays can also be determined. For the dataset used, the AR CALUX

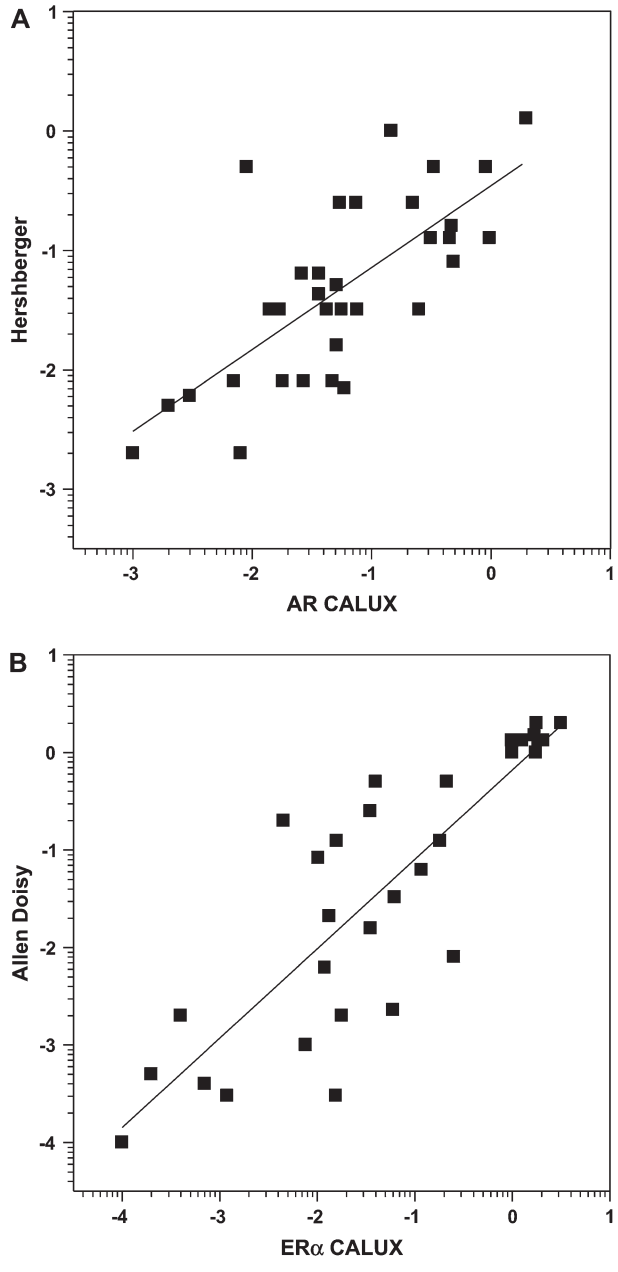

FIG. 4. Comparison of *in vitro* CALUX reporter gene assays with the *in vivo* Hershberger and Allen-Doisy assays for determination of androgenic and estrogenic activity of various compounds. Relative Agonistic Activities (RAAs) of various androgenic or estrogenic compounds (see Tables 1 and 2) were determined by AR and ERα CALUX reporter gene assays and compared with the RAAs obtained with the Hershberger androgenic assay (A) and the Allen-Doisy estrogenic assay (B), respectively.

reporter gene assay is most related to the AR binding assay and the *in vivo* Hershberger assay clusters separately from the other *in vitro* assays (Fig. 5A). For the estrogenic compound dataset, different assay relationships are observed. Here, the ERα CALUX reporter gene assay is most related to the *in vivo* Allen-Doisy assay, while the ER binding assay clusters separately from the other assays (Fig. 5B). Furthermore, it is obvious that the similarity between the different estrogen reporter assays is higher than for the androgen reporter assays, indicated by the small distance between the individual estrogen assays and the larger distance between the Hershberger assay and the other androgen assays (Fig. 5).

DISCUSSION

To get insight in the robustness of the *in vitro* data set, we have made an extensive comparison between assays used to determine androgenicity and estrogenicity. Excellent correlations were found between different reporter gene assays, and these results were also very comparable with the data of the receptor binding assays. These results are remarkable, since we used different reporter gene assays at different locations, and different experimental protocols. This demonstrates the robustness of the currently used reporter gene assays when carried out in experienced laboratories. The intra-laboratory CVs that we calculated for the different reporter gene assays of 12–25% compare well with previously published data of other laboratories (Andersen *et al.*, 1999; Körner *et al.*, 2004).

An illustration is provided how to handle a large steroid compound data set obtained with various bioassays allowing the cross-assay bioactivity profiling of a given compound. By using a hierarchical clustering algorithm, activity relationships between compounds and also relationships between various bioassays (as discussed previously) can be determined. In this study the dataset of androgenic or estrogenic compounds is rather limited, but already interesting relationships in both directions (compounds and assays) were observed. This way of data handling proves promising for building a compound activity database which can be used to profile the activity of new (pharmaceutical) compounds and even to determine the identity of unknown compounds present in various matrices, such as blood/urine (clinical and sport doping applications), food/feed, and environmental samples. The success of the latter option depends greatly on the number of compounds and assays within the database.

Comparison of the data of the reporter gene assays with receptor binding assays again revealed a good correlation, confirming that nuclear receptor binding is a critical step in steroid action. The receptors used in all of our assays are of human origin, explaining the high levels of concordance between the data. The fact that we used reporter gene assays in which the receptors were stably introduced under control of a constitutive promoter has likely contributed to the robustness of the response. Expectedly, antagonists scored negative in reporter gene assays while being positive in receptor binding assays. The characteristic of binding agonists as well as antagonists is a clear drawback of binding assays, since many compounds have been found that can act antagonistically toward the androgen and estrogen receptor. For example, it has been found that a wide variety of environmental chemicals

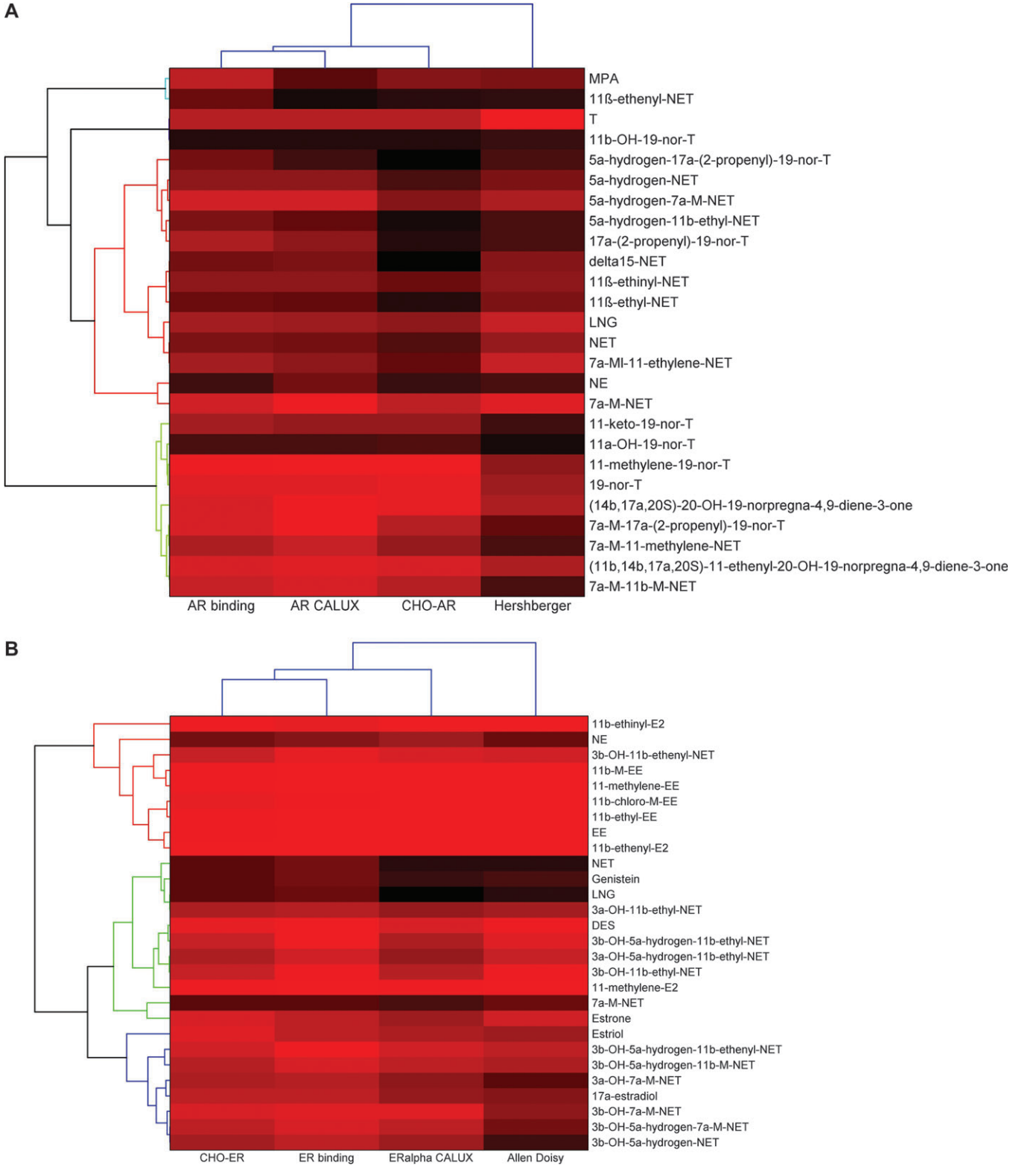

FIG. 5. Hierarchical clustering of androgenic and estrogenic compounds. Relative Agonistic Activities (RAAs) of androgenic or estrogenic compounds (see Tables 1 and 2) determined by all used assays were clustered according to the hierarchical clustering method. (A) Androgenic compounds ($n = 26$) in relation to the androgenic assays (AR CALUX and CHO-AR reporter gene assays, AR binding and Hershberger assay). (B) Estrogenic compounds ($n = 30$) in relation to the estrogenic assays (ERα CALUX and CHO-ERα reporter gene assays, ER binding and Allen-Doisy assay). The intenser the red color, the higher the activity of the compound. Black means no activity.

have weak anti-androgenic activity (Schreurs *et al.*, 2005; Sonneveld *et al.*, 2005). In the reporter gene assays used, antagonistic activity can easily be dissected from agonistic activity by changing the experimental setting (Schoonen *et al.*, 2000a; Sonneveld *et al.*, 2005).

Although the correlation between the results of the different *in vitro* assays was very strong, expectedly the correlation with the *in vivo* data was weaker, as is generally experienced when trying to mimic the complex physiology in whole animals using *in vitro* systems. Although the *in vitro* data showed a very good correlation with the Allen-Doisy test, which measures vaginal cornification in ovariectomized rats (this study; de Gooyer *et al.*, 2003; Schoonen *et al.*, 2000a), as was also demonstrated earlier by Yamasaki *et al.* (2002) for a HeLa cell line derived ERα reporter gene cell line and the rat uterotrophic assay, the correlation with the Hershberger test was weaker (this study; Schoonen *et al.*, 2000a,b). This mainly seems to be related to the difference in sensitivity between both *in vivo* assays. While the physiological dose for an active estrogen ranges from 10 to 100 pM, the physiological dose for an active androgen ranges from 0.2 to 10 nM. This means that for the Allen-Doisy assay a much lower amount of estrogenic compound can be applied to the animal to activate the estrogen receptor (e.g., 0.5 μg/kg of 17β-estradiol) than for androgens activating the AR in the Hershberger assay (160 μg/kg of testosterone), For the weaker androgens, the activating dose might even not be reached. It should be noted that in this study we have restricted ourselves to measuring relatively potent activities of compounds with a pharmacological interest, using relatively low maximal doses of compounds. This might imply that, in other fields such as chemical risk analysis of chemicals, using relatively weaker compounds, the Hershberger assay might not be very suitable as a test system (Charles *et al.*, 2005), while *in vivo* estrogen assays are, as shown recently for bisphenol A and closely related compounds (Kitamura *et al.*, 2005).

In our hands, in both assays the response of individual compounds *in vitro* was almost without exception able to predict the *in vivo* activity with an uncertainty of a factor 10 or less in either direction, suggesting that, overall, the correlation between the two types of *in vivo* tests with their corresponding *in vitro* tests will not be very different when expanding the dataset. This ability to predict the *in vivo* activity of chemicals within a factor 10 difference seems satisfactory for a number of applications. An important requirement in activity screens, particularly when related to risk analysis is a low level of false-negative predictions. Because of the high sensitivity of the *in vitro* screens, these false-negatives can easily be avoided even when taking an uncertainty factor of 10–100 into account.

There may be various reasons for the differences between the *in vitro* and *in vivo* data. First of all, since the nuclear receptors are the most important determinants of steroid action, variations in binding characteristics will change the outcome of an assay. Since there are species differences between the receptors used in different assays, being human derived in the *in vitro* assays and rodent in the *in vivo* assays, this might have contributed to differences in responses. However, it is unlikely that these species differences contribute to a large extent to the differences between *in vitro* and *in vivo* data, since the steroid receptors and their binding characteristics are very well conserved between species, and certainly between mammals (Escriva *et al.*, 2004; Owen and Zelent, 2000). However, the presence/absence of specific cofactors (coactivators and corepressors) for the receptors may account for subtle differences between the results of the different assays in a way that is difficult to predict. A major source of differences may be the differences in metabolism and pharmacokinetics, involving adsorption, distribution (including binding to general and steroid-specific binding proteins), and excretion of steroids in the different assay systems. All these latter mechanisms will generally be underrepresented in an *in vitro* system. Since our test compounds were mainly steroidal, specific metabolic conversions could have occurred that have the potential to greatly influence their hormonal activity. Steroid hormones undergo active metabolism through specific enzymatic conversions such as activating CYP17, CYP19, 3α, 3β-, and 17β-HSD, and 5α-reductase activities (Auchus, 2004; Soronen *et al.*, 2004) and inactivating glucuronidase and sulfatase activities (Basinska and Florianczyk, 2003), leading to compounds with altered biological activity. In fact, there are many indications that metabolism is an important source of differences between the *in vitro* and *in vivo* assays, as is illustrated below.

The activity of DHT was equal to that of testosterone *in vivo,* in contrast to *in vitro,* where DHT was the more active compound. The latter observation is commonly accepted for *in vitro* AR reporter gene assays (de Gooyer *et al.*, 2003; Sonneveld *et al.*, 2005; Wilson *et al.*, 2002). An explanation for the observation that testosterone seems to be favored in the Hershberger assay can be that the metabolic clearance *in vivo* is faster for DHT (Kumar *et al.*, 1999), due to 3α and 3β-hydroxylation, glucuronidation, and sulfation, besides only 17β-HSD activity acting on testosterone, resulting in testosterone as the abundant androgen in serum and *net* similar results for testosterone and DHT in the Hershberger assay. Additionally, *in vitro* 5α-reductase activities in U2-OS and CHO cells might be low compared to the *in vivo* situation. Surprisingly, propionate modifications at R17 are much less potent in CHO cells compared to testosterone. In U2-OS cells and *in vivo* this clearly was not the case, suggesting a relatively low esterase activity in CHO cells. This suggestion is strengthened by the fact that EE and its derivatives are also more potent in U2-OS and the *in vivo* Allen-Doisy compared to CHO cells.

Specific structure/activity relationships were noted for androgens as well as estrogens from modifications on various positions within the androgen or estrogen molecule included in this study (see Tables 1 and 2). Some of the modifications clearly influenced the relative potency *in vitro* and *in vivo* of the compounds. For example, comparing testosterone with its

19-nor derivatives nandrolone (19-nor-testosterone), 11β-methyl-19-nor-testosterone, and 11-methylene-19-nor-testosterone, the 19-nor compounds are 3 to 12 times more potent than testosterone in *in vitro* binding and reporter gene assays, but around 15-fold weaker than testosterone in the *in vivo* Hershberger assay. This *in vivo* difference might be due to the fact that, in the accessory sex organs (e.g., the prostate), testosterone is 5 alpha-reduced to DHT, which, due to its higher affinity for AR, amplifies the action of testosterone. In contrast, when 19-nor-testosterone is 5 alpha-reduced its affinity for AR decreases, resulting in a decrease in its androgenic potency (Kumar *et al.*, 1999; Sundaram *et al.*, 1995). Addition of a large substituent to 19-nor-testosterone at the 17α-position, like an ethinyl (norethisterone: NET) or propenyl (17α-(2-propenyl)-19-nor-testosterone), sharply reduces the androgenic activity *in vitro,* while the *in vivo* activities of these compounds remain the same as for nandrolone. This latter might be due to the higher *in vivo* stability of these compounds, as is the case for 17α-ethinyl-E2 in comparison to E2 in the ER bioassays (Fotherby, 1996). On the other hand, a small methyl addition to the 6α-, 7α-, 11-, 11β-, or 17α-position is allowed without a tremendous reduction in potency *in vitro* and *in vivo,* with the exception of the 7α-methyl addition (MENT) resulting in an increase in *in vitro* (4-fold compared to nandrolone) and *in vivo* (16-fold compared to nandrolone) activities. The high potency of MENT is primarily related to its higher affinity to AR as was proposed earlier by Kumar *et al.* (1999) and shown by AR binding in this study. As expected for hydroxylated steroids, a polar group like 11α-OH or 11β-OH reduces the activity by at least 50-fold for 19-nor-testosterone derivatives, *in vitro* as well as *in vivo.*

In U2-OS cells 17β-HSD activity is also present, suggested by the activity of the testosterone precursor androstenedione in the AR CALUX reporter gene assay (this study) and RT-PCR experiments (data not shown), while binding to the AR is relatively low. Unfortunately, no CHO and Hershberger data are present for this compound.

Another notable exception was progesterone being negative *in vitro,* but active in the Hershberger assay. Progesterone is generally not considered to have strong androgenic activity, but in the Hershberger assay possibly is converted to active androstenedione/testosterone. In addition, MPA and LNG-(acetate) were also positive androgenic *in vivo* progestagenic compounds, suggesting that other progestagenic compounds are also active in the Hershberger assay (as demonstrated by Schoonen *et al.*, 2000a,b). Other hormones that were suggested to be possibly active in the Hershberger assay are estrogens. Clearly, ERβ is highly expressed in the prostate, and inhibition of ventral prostate proliferation is regulated by ERβ ligands, while loss of ERβ is associated with tumor progression (Bardin *et al.*, 2004; Imamov *et al.*, 2004). The fact that other hormones than androgens have effects in the Hershberger assay indicate that the *in vivo* assays are never entirely specific (Zacharewski, 1998).

Metabolic activities, combined with nonselectivity of the *in vivo* assays may lead to surprising results. For example 3-hydroxy substitution of androgens (due to 3α- and 3β-HSD activity) leads to inactive androgens, while estrogenic compounds can be converted to progestagens, especially in case of the NET derivatives. For progestagens, it is known that they can inhibit the estrogenic activity in the Allen-Doisy test (Schoonen *et al.*, 2000b). Thus if a compound is metabolized into active progestagens, only measurement in the presence of an anti-progestagen allows the correct *in vivo* determination of the estrogenic activity. Within Organon, examples for 11β-ethyl-NET and 11β-ethenyl-NET are available, in which these compounds are much more potent in an Allen-Doisy test in combination with an anti-progestagen than when given alone (data not shown). Taken together, the observed results suggest that metabolism, combined with relative nonspecificity of the *in vivo* assays can lead to differences between *in vitro* and *in vivo* assays.

Our results suggest that inclusion of ADME could further improve the predictions made by the *in vitro* models. It should be noted, however, that these predictions can never be absolutely exact. This situation is not different in the *in vivo* models where species and strain differences have been reported in the response to hormonal agents that can reach two to three orders of magnitude (Hengstler *et al.*, 1999; Spearow *et al.*, 1999, 2001). Even within an organism, the response of different target organs to hormones may vary strongly, which has been shown elegantly using transgenic mice expressing reporter gene constructs (Ciana *et al.*, 2003; Lemmen *et al.*, 2004). In this light the *in vitro* predictions, deviating a factor 10 from the *in vivo* data are remarkably accurate, especially because different species are used in the models. *In vitro* models have a clear advantage of being able to use human materials either directly applied on the reporter cells (e.g., plasma or serum; Sonneveld *et al.*, 2005) or extracted (e.g., urine; Legler *et al.*, 2002). This, however, also poses a problem when validating such models. When trying to further improve the *in vitro* models, one of the difficulties will be the absence of sufficient appropriate *in vivo* determinations, since these ideally are human clinical data.

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